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REMARKS

In the Specification:

Applicant thanks the Examiner for withdrawing the objections to the specification raised in the Office Action of March 24, 2003.

In the Claims:

Claims 25-27 have been amended, per the Examiner's suggestion, by replacing "it" in subpart (d) with "its" to be grammatically correct.

Claims 27-31 have also been amended, per the Examiner's suggestion, by amending "of" to read "encoding" to clarify that the nucleic acid referred to in the claim encodes the polypeptide shown in Figure 20.

Claims 22-26 have further been amended to reflect that the claimed nucleic acid is amplified in lung and/or colon tumors.

Claim 26 has been amended to clarify that it refers to the nucleic acid of claim 22 encoding a polypeptide.

The Examiner has allowed claims 32-34. Therefore, Applicant has rewritten claims 32-34 in independent format, thereby removing the dependency from rejected claim 27.

Claims 22-24, and 35 are canceled herein without prejudice or disclaimer.

Claim 38 is amended so that it does not improperly depend from a canceled claim.

New claims 42 and 43 have been added. New claims 42 and 43 do not encompass new matter and are supported at pages 59-62 of the specification.

Response to Amendment:

Applicant notes that the Examiner agrees that the declaration of Audrey D. Goddard, Ph.D., under 37 CFR 1.132, filed June 26, 2003, overcomes the rejection of claims 22-41 for lack of utility under 35 U.S.C. §101.

Priority Determination:

The Examiner has found that the present application has an effective priority date of 12/22/98, based on the filing date of U.S. Provisional Application Serial Number 60/113,296.

Claim Rejections:

35 U.S.C. § 102(e)

The Examiner has rejected claims 22-27, 31, 35 and 38-41 under 35 U.S.C. § 102(e) as being anticipated by Holtzman *et al.*, U.S. Patent Application Publication US20020028508, effective filing date, April 23, 1998. Specifically, the Examiner alleges that Holtzman *et al.* disclose a nucleic acid sequence that is 94.1% identical to the nucleic acid molecule of SEQ ID NO:49, as well as a protein that is 96.8% identical to the protein of SEQ ID NO:50.

Applicant respectfully disagrees that Holtzman *et al.* anticipates the claimed invention. First, "[t]o serve as an anticipating reference, the reference must enable that which it is asserted to anticipate. 'A claimed invention cannot be anticipated by a prior art reference if the allegedly anticipatory disclosure cited as prior art is not enabled.'" *See Elan Pharm., Inc. v. Mayo Found. For Med. Ed. and Research,* 2003 U.S. App. LEXIS 20195 (Fed. Cir. 2003) citing *Amgen, Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1354 (Fed. Cir. 2003). Applicants submit that Holtzman *et al.* is not enabled because the nucleic acid and protein cited by the Examiner (hereinafter collectively referred to as "T139") are not supported by either a specific and substantial utility or a well-established utility.

Specifically, Holtzman *et al.* discloses both the nucleic and amino acid sequences of T139 and reference a deposit of a cDNA (ATCC 98694). As the Examiner notes, the nucleic acid sequence disclosed by Holtzman *et al.* is 94.1% identical to the nucleic acid molecule of SEQ ID NO:49 and the amino acid sequence disclosed by Holtzman *et al.* 96.8% identical to the protein of SEQ ID NO:50. Much like the present specification, Holtzman *et al.* discloses various characteristics of the cDNA sequence encoding T139, as well as various predicted characteristics of the T139 protein (p. 8, paragraph 0107). Also much like the present specification, Holtzman *et al.* discloses that sequence analysis revealed various homologies, for example T139 is described as homologous to testis-specific protein-1 (TPX-1) (page 8, paragraph 0110). Holtzman *et al.* also explains generally various variant T139 sequences, antibodies to T139, assays, and methods of treatment. In addition, Holtzman *et al.* discloses the isolation and characterization of T139 cDNA, the distribution of T139 mRNA in human tissues and the predicted characterization and production of T139 proteins. However, unlike the present specification, Holtzman *et al.* does not enable one of skill in the art to use T139. Specifically, although Holtzman *et al.* generally notes that T139 might be used to modulate the function, morphology, proliferation and/or differentiation of cells in tissues in which it is expressed (p. 39, paragraph 0367), or used to treat renal (kidney) disorders (p.39-40, paragraph 0375), or used to treat testicular disorders (p. 40, paragraph 0376), Holtzman *et al.* does not disclose any working example of a credible, specific and substantial utility for T139. Hence, Applicant submits that Holtzman *et al* does not enable that which it is asserted to anticipate and therefore, does not anticipate the present invention.

In addition, "[a] claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). MPEP § 2131.01. Holtzman *et al.* neither explicitly nor inherently discloses nucleic acids that are amplified in lung and/or colon tumors and therefore, cannot anticipate these claims.

Holtzman *et al.* describes T139 as being homologous to testis-specific protein (see p. 8, paragraph 0110) and as playing a role in kidney defects such as kidney failure or hyperplasia (see p. 38, paragraph 0359). However, Holtzman *et al.* does not disclose or examine the presence of T139 nucleic acid in lung or colon tumor tissue. Instead, at page 8, paragraph 0113, Holtzman *et al.* discloses T139 is expressed at high levels in the kidney with lower levels in the testis but that "[n]o T139 expression was observed in the heart, brain, placenta, **lung**, liver, skeletal muscle, pancreas, spleen, thymus, ovaries, small intestine, **colon** and peripheral blood leukocytes." (emphasis added). *See also* p. 41, paragraph 0397. Thus, for this additional reason, Holtzman *et al.* does not anticipate the present claims, which are directed to nucleic acids that are amplified in lung and colon tumors. Hence, Applicant has overcome this ground of rejection for claims 25-27, 31, 35 and 38-41 and respectfully requests that it be withdrawn.

35 U.S.C. § 112, second paragraph

Claims 22-27, 31, 35, and 38-41 are rejected under 35 U.S.C. § 112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. Specifically, the Examiner contends that the recitation of "the extracellular domain" . . . lacking its associated signal sequence" is indefinite as a signal sequence is not generally considered to be part of an extracellular domain, as signal sequences are cleaved from said domains in the process of secretion from the cell.

Applicant respectfully disagrees that the claims are indefinite. As taught by the Alberts *et al.* textbook, Molecular Biology of the Cell, 3rd edition, (pp. 557-560; Appendix A), a signal sequence is not *necessarily* cleaved from the extracellular domain of a protein, even though a "signal peptide is often (but not always) removed from the finished protein...." *Id.* For example, in polypeptides with both signal sequences and distant transmembrane domains, the polypeptide can exist as a transmembrane polypeptide with its signal sequence uncleaved. The signal sequence is later cleaved with the transmembrane domain influencing the cleavage process. *See e.g.,* Rehm et al., EMBO J. 7:1573-1582 (April 2, 2001) (Appendix A). Depending on further processing, the

polypeptide can either later be released as a soluble polypeptide or remain a transmembrane polypeptide. Thus, Applicant respectfully submits that the art *does* recognize that a signal sequence can be part of an extracellular domain.

The Examiner maintains her rejection of claim 35 under 35 U.S.C. § 112, ¶2, alleging that the claim is indefinite because page 30 of the specification sets forth several different hybridization conditions exemplified by high stringency conditions. The Examiner kindly notes that this rejection can be overcome by reciting one of the hybridization conditions recited in the specification, such as the one recited at page 18 of the Amendment and Request for Reconsideration mailed June 24, 2003, as well as the high-stringency wash conditions. Applicant has canceled claim 35 herein without prejudice or disclaimer. Accordingly, Applicant respectfully requests that the rejections of claims 25-27, 31, 35, and 38-41 under 35 U.S.C. § 112 ¶2 for indefiniteness be withdrawn.

35 U.S.C. § 112, first paragraph

Enablement

Claims 22-31, 35, and 38-41 remain rejected under 35 U.S.C. § 112, first paragraph for lack of enablement because the Examiner contends that although the specification enables the nucleic acid molecule having the nucleotide sequence of SEQ ID NO:49, or fragments of the nucleic acid sequence, it does not enable polynucleotides encoding the protein of SEQ ID NO:50 or encoding proteins sharing at least 80% sequence identity with the protein of SEQ ID NO:50. Therefore, the Examiner concludes that the scope of enablement is not commensurate with the scope of the claims. Specifically, the Examiner notes that claims 22-31, 35, and 38-41 encompass nucleic acids encoding polypeptides, and because of the degeneracy of the genetic code, the Examiner contends that such nucleic acids could deviate significantly from the nucleic acid of SEQ ID NO:49. The Examiner further asserts that nucleic acids encoding polypeptides having at least 80% sequence identity to the protein of SEQ ID NO:50 would deviate even more so from the nucleic acid of SEQ ID NO:49. Based on this argued deviation, the Examiner concludes that such nucleic acids would not be useful as diagnostic markers.

Applicant respectfully disagrees. Each of the claims requires that the claimed nucleic acid be amplified in lung and/or colon tumors. Therefore, any nucleic acid within the scope of the claims would be useful as a diagnostic marker. Further, at page 59, line 13 to page 63, line 36, the specification enables one of skill in the art to make variant sequences of SEQ ID NO:49 without undue experimentation. The specification also discloses Example 28 at pages 119-137, which describes several assays that can be used to determine whether any variant nucleic acid is amplified in lung or colon tumor tissue and thus, within the scope of the claims. Hence, Applicant respectfully requests the Examiner withdraw this ground of rejection.

The Examiner further rejects claims 22-31, 35, and 38-41, alleging that although the data in the specification shows that gene copy number is increased in certain tumor tissue samples, it is not predictable that the PRO347 polypeptide is overexpressed in any tumor cell in which the encoding nucleic acid is amplified.

Applicant believes the Examiner's rejection of claims 22-31, 34, and 38-41, which are directed to *nucleic acids,* based on unpredictability of *polypeptide* overexpression is improper and requests that it be withdrawn. First, Applicant has amended the claims to clarify that it is the claimed nucleic acid that is amplified in lung and/or colon tumors. Second, overexpression of the encoded protein is irrelevant to the diagnostic utility of the claimed *nucleic acid* because the nucleic acid is able to function as a diagnostic regardless of whether the encoded protein is overexpressed. Applicant's utility in this application is based on the diagnostic utility of nucleic acids. Applicant does not claim any utility in this application, based on protein overexpression.

In any event, Applicant respectfully disagrees with the Examiner that it is not predictable that PRO347 polypeptide is overexpressed in any tumor cell in which the encoding nucleic acid is amplified and therefore, one of skill in the art would not be enabled to use the present invention. MPEP §2164.03 states that "'[t]he predictability or lack thereof' in the art refers to the ability of one skilled in the art to extrapolate the disclosed or known results to the claimed invention. If one skilled in the art can readily anticipate the effect

of a change within the subject matter to which the claimed invention pertains, then there is predictability in the art." Applicant discloses in the specification at page 119, lines 27-30, that in the art, the general presumption is that gene amplification is associated with overexpression of the gene product. Thus, it is predictable that a nucleic acid encoding a gene that is amplified in tumor tissues would also encode a polypeptide that is overexpressed in tumor tissues and therefore, one of skill in the art would know that such polypeptides would have utility as diagnostic markers for determining the presence of tumor cells in lung and colon tissue samples. Moreover, one of skill in the art would know the claimed nucleic acid sequences have utility as sources of nucleic acid probes for carrying out diagnostic procedures based on gene product overexpression.

In addition, one of skill in the art would know that the claimed nucleic acids and encoded polypeptides have significant utility even in the absence of overexpression of gene product. Enclosed herein is a second declaration. This second declaration is made by Avi Ashkenazi, Ph.D. (*see* Appendix B), an expert in the field of cancer biology and an inventor of the present invention. As Dr. Ashkenazi explains at paragraph 5 of his declaration:

> "[e]ven in the absence of over-expression of the gene product, amplification of a cancer marker gene is useful in the diagnosis or classification of cancer, or in predicting or monitoring the efficacy of cancer therapy."

Dr. Ashkenazi further explains the relationship between the gene amplification data presented in the specification and gene product (protein) expression in paragraph six stating:

> "the very absence of gene product over expression . . . provides significant information . . . . (For example,) . . . if over-expression of the gene product does not parallel gene amplification in certain tumor types but does so in others, then parallel monitoring of gene amplification and gene product over-expression enables more accurate tumor classification and hence better determination of suitable therapy."

Dr. Ashkenazi also points out that absence of over-expression of gene product is "crucial information" for the practicing clinician when determining which agents to use in treating a patient. For example, in such a situation agents that target gene product would not be effective. Hence, Applicant submits that one of skill in the art is enabled to practice the claimed invention and respectfully requests that the rejection of claims 25-31, 35, and 38-41 be withdrawn.

Written Description

Claims 25-26, 35, and 38-41 are rejected under 35 U.S.C. 112, first paragraph for failure to satisfy the written description requirement. The Examiner alleges that Claims 25-26, 35, and 38-41 contain subject matter which is not described in the specification in such a way as to reasonable convey to one skilled in the art that the inventor, at the time of the application, had possession of the claimed invention. The Examiner maintains the previous rejection based on an alleged lack of written description because the Examiner contends that amplification of a nucleic acid does not necessarily result in an increase in expression of the encoded polypeptide.

Applicant respectfully disagrees and submits that Claims 25-26, 35 and 38-41 are adequately described in the present application. First, as mentioned above, Applicant has clarified that the claimed nucleic acid is amplified in lung and/or colon tumors. Second, as the Examiner notes, the written description requirement requires that an applicant's specification convey with reasonable clarity to those skilled in the art, that as of the filing date sought, he or she was in possession of the invention. *Vas-Cath, Inc. v. Mahurkar,* 19 USPQ2d 1111, 1116 (Fed. Cir. 1991). Moreover, in order to have possession of members of a claimed genus, the specification ***need not*** describe all of the species that the genus encompasses. *Amgen Inc. v. Chugai Pharmaceutical Co.*, 18 USPQ2d 1016, 1027 (Fed. Cir. 1991). Applicant has demonstrated possession of the claimed invention, at least by disclosure of SEQ ID NOS: 49 and 50, as well as by deposit of DNA44176-1244 on December 10, 1997 as disclosed on page 148 of the specification (ATCC deposit no. ATCC209532).

Further, compliance with the written description requirement does not require an applicant to describe exactly the subject matter claimed; rather, the description must clearly allow a person of ordinary skill in the art to recognize that he or she invented what is claimed. *Vas-Cath Inc. v. Mahurkar*, 19 USPQ2d 1111, 1116 (Fed. Cir. 1991). The analysis for determining whether the present specification provides written description support for the invention defined by claims 25-26, 35 and 38-41 may be performed by numerous methods, several of which are described in the Guidelines and further exemplified in the Revised Interim Written Description Guidelines Training Materials ("Written Description Training Materials"), published on the USPTO website at http://www.uspto.gov/web/offices/pac/writtendesc.pdf. These Written Description Training Materials are designed to provide additional clarity to the Guidelines which are published in the Federal Register, Volume 66, No. 4, pages 1099-1111. In fact, as indicated in the USPTO press release of March 1, 2000 introducing the Written Description Examination Training Materials (Press Release #00-15), these training materials were promulgated by the USPTO and are:

> "designed to aid PTO's patent examiners in applying the interim written description and utility guidelines in a uniform and consistent manner to promote the issuance of high quality patents. The training materials will also assist patent applicants in responding to the PTO when utility or written description issues are raised during the examination of a patent application." (emphasis added)

With regard to claims 25-26, 35, and 38-41, the present situation is analogous to Example 14 on pages 53-55 of the Written Description Training Materials. More specifically, in Example 14 on pages 53-55 of the enclosed Written Description Training Materials, a claim directed to a protein and variants thereof having 95% sequence identity, all of which share the same biological function, is analyzed for its compliance with the written description requirement of 35 U.S.C. § 112, first paragraph. The Written Description Training Materials conclude that such a claim satisfies the written description requirement of 35 U.S.C. § 112, first paragraph, when (1) a single protein sequence is actually reduced to practice, (2) procedures for making variants of that "reduced to practice" protein sequence are conventional in the art, and (3) an assay is described which allows identification of other proteins having the same biological

activity. The reasoning provided by the USPTO in the Written Description Training Materials is that:

> "[t]here is actual reduction to practice of the single disclosed species. The specification indicates that the genus of proteins that must be variants of SEQ ID NO:...does not have substantial variation since all of the variants must possess the specified [biological function] and must have at least 95% identity to the reference sequence, SEQ ID NO:...The single species disclosed ***is representative of the genus*** because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO:...which are capable of the specified [biological function]. One of skill in the art would conclude that applicant was in possession of the necessary common attributes possessed by members of the genus.....{As such], the disclosure meets the requirements of 35 U.S.C. § 112, first paragraph, as providing adequate written description for the claimed invention." (emphasis added).

Analogous to Example 14 of the Written Description Training Materials, the present specification discloses and actually reduces to practice a nucleic acid recited in claims 25-26, 35 and 38-41 (*i.e.*, SEQ ID NO:49) as well as a polypeptide encoded by that nucleic acid (*i.e.*, SEQ ID NO:50). Moreover, the nucleic acid variants encompassed within claims 22-26, 35 and 38-41 ***do not have substantial variation*** with SEQ ID NO:49 because (a) they share at least 95% sequence identity with SEQ ID NO:49 or the encoded polypeptide (SEQ ID NO:50) (Applicants note that methods for routinely determining nucleic acid and/or amino acid sequence identity are described in detail in the present specification at page 23, line 34 to page 29, line 2, *see also* pages 34-54), and (b) they share the biological function of being amplified in lung and/or colon tumors. (Applicants note that the specification describes in detail in Example 28 an assay that is useful for identifying nucleic acids encoding polypeptides having this biological function). As such, the nucleic acids encompassed within claims 25-26, 35, and 38-41 all share substantial common structural features (*i.e.*, at least 95% sequence identity)

and substantial common functional features (*i.e.,* being amplified in lung and/or colon tumors). Moreover, the present specification also describes conventionally known methods used and known in the art for preparing a multitude of variants (see the present specification at page 59, line 13 to page 63, line 36).

In view of the legal standard regarding the written description requirement under 35 U.S.C. § 112, first paragraph, in combination with the interpretation of the written description requirement by the United States Patent and Trademark Office as set forth in the Guidelines, and given the above, Applicants respectfully submit that currently pending claims 25-26, 35 and 38-41 satisfy the written description requirement of 35 U.S.C. § 112, first paragraph because it would be clear to one of skill in the art that Applicant possessed the claimed subject matter at the time of filing the instant application.

Moreover, claims 25-26 are analogous to the claim found to satisfy the written description requirement in Example 14 of the enclosed Written Description Training Materials. As such, under the Guidelines and the examination training materials promulgated by the USPTO for ensuring consistent examination of written description compliance during prosecution of patent applications, the written description requirement of 35 U.S.C. § 112, first paragraph, is satisfied for claims 25-26, 35 and 38-41. Therefore, Applicant respectfully requests this ground of rejection be withdrawn.

Claims 38-41 are directed to a vector comprising the nucleic acid of Claim 25. Applicant submits that the nucleic acid of Claim 25 is adequately described as discussed above. Vectors, host cells and cell transformation are described at pages 64-68. Specifically, Applicant describes production of PRO by culturing host cells transformed or transfected with a vector containing PRO nucleic acid. Even further, Applicant describes the expression of PRO polypeptides in *E.coli*, mammalian cells (CHO cells), and yeast on pages 111-115. The skilled artisan would understand that the described procedures could be carried out using PRO347.

For all these reasons, Applicant submits that the specification recites distinguishing, identifying characteristics, sufficient to satisfy the written description requirement with respect to claims 25-26, 35, and 38-41. Applicant respectfully requests the Examiner withdraw this ground of rejection.

SUMMARY

Applicant thanks the Examiner for noting that currently pending Claims 32-34 are allowable. Applicant believes that currently pending Claims 22-31 and 38-41 are also patentable. Applicant respectfully requests the Examiner grant early allowance of this application. The Examiner is invited to contact the undersigned attorney for Applicant via telephone if such communication would expedite this application.

Respectfully submitted,

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MOLECULAR BIOLOGY OF
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Front cover: The photograph shows a rat nerve cell in culture. It is labeled (*yellow*) with a fluorescent antibody that stains its cell body and dendritic processes. Nerve terminals (*green*) from other neurons (not visible), which have made synapses on the cell, are labeled with a different antibody. (Courtesy of Olaf Mundigl and Pietro de Camilli.)

Dedication page: Gavin Borden, late president of Garland Publishing, weathered in during his mid-1980s climb near Mount McKinley with MBoC author Bruce Alberts and famous mountaineer guide Mugs Stump (1940–1992).

Back cover: The authors, in alphabetical order, crossing Abbey Road in London on their way to lunch. Much of this third edition was written in a house just around the corner. (Photograph by Richard Olivier.)

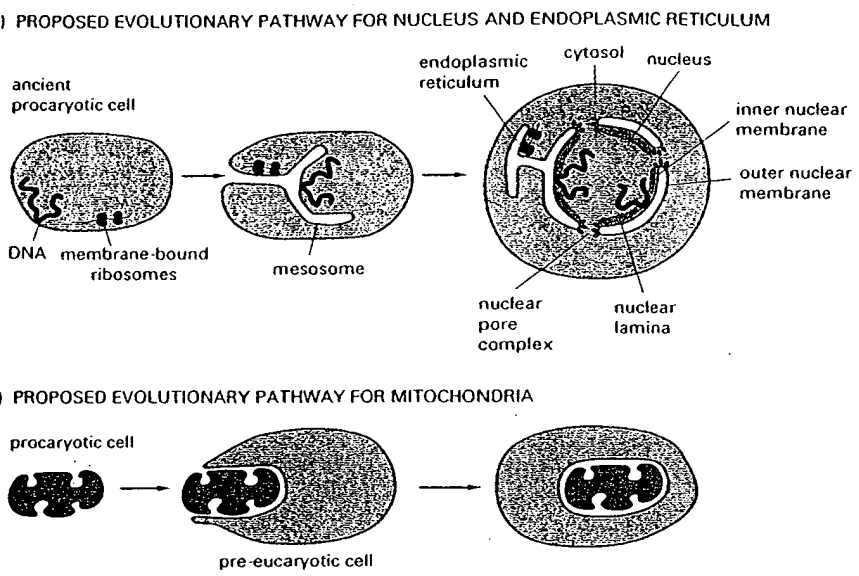


Figure 12–5 Hypotheses for the evolutionary origins of some membrane-bounded organelles. The origins of mitochondria, chloroplasts, ER, and the cell nucleus could explain the topological relationships of these intracellular compartments in eucaryotic cells. (A) A possible pathway for the evolution of the cell nucleus and the ER. In some bacteria the single DNA molecule is attached to an invagination of the plasma membrane, called a *mesosome*. Such an invagination in a very ancient procaryotic cell could have spread to form an envelope around the DNA while still allowing access of the DNA to the cell cytosol (as is required for DNA to direct protein synthesis). This envelope is presumed to have eventually pinched off completely from the plasma membrane, producing a nuclear compartment surrounded by a double membrane. As illustrated, the nuclear envelope is organized by a fibrous shell called the *nuclear lamina* and is penetrated by communicating channels called *nuclear pore complexes*. Because it is surrounded by two membranes that are in continuity where they are penetrated by these pores, the nuclear compartment is topologically equivalent to the cytosol. The lumen of the ER is continuous with the space between the inner and outer nuclear membranes and topologically equivalent to the extracellular space. (B) Mitochondria (and chloroplasts) are thought to have originated when a bacterium was engulfed by a larger pre-eucaryotic cell. They retain their autonomy. This may explain why the lumens of these organelles remain isolated from the vesicular traffic that interconnects the lumens of many other intracellular compartments.

plasma membrane of the bacterium, while the lumen of these organelles evolved from the bacterial cytosol. As might be expected from such origins, these two organelles remain isolated from the extensive vesicular traffic that connects the interiors of most of the other membrane-bounded organelles to one another and to the outside of the cell.

This evolutionary scheme groups the intracellular compartments in eucaryotic cells into five distinct families: (1) the nucleus and the cytosol, which communicate through the nuclear pores and are thus topologically continuous (although functionally distinct); (2) all organelles that function in the secretory and endocytic pathways—including the ER, Golgi apparatus, endosomes, lysosomes, and numerous classes of transport vesicles; (3) the mitochondria; (4) the plastids (in plants only); and (5) the peroxisomes (whose evolutionary origins are discussed later).

Proteins Can Move Between Compartments in Different Ways [3]

All proteins begin being synthesized on ribosomes in the cytosol, except for the few that are synthesized on the ribosomes of mitochondria and plastids. Their subsequent fate depends on their amino acid sequence, which can contain **sorting signals** that direct their delivery to locations outside the cytosol. Most proteins do not have a sorting signal and consequently remain in the cytosol as permanent residents. Many others, however, have specific sorting signals that direct their transport from the cytosol into the nucleus, the ER, mitochondria, plastids (in plants), or peroxisomes; sorting signals can also direct the transport of proteins from the ER to other destinations in the cell.

To understand the general principles by which sorting signals operate, it is important to distinguish three fundamentally different ways by which proteins move from one compartment to another. (1) The protein traffic between the cytosol and nucleus occurs between topologically equivalent spaces, which are in continuity through the nuclear pore complexes. This process is called **gated transport** because the nuclear pore complexes function as selective gates that can actively transport specific macromolecules and macromolecular assemblies, although they also allow free diffusion of smaller molecules. (2) In **transmembrane transport** membrane-bound *protein translocators* directly transport specific proteins across a membrane from the cytosol into a space that is topologically distinct. The transported protein molecule usually must unfold in order to snake

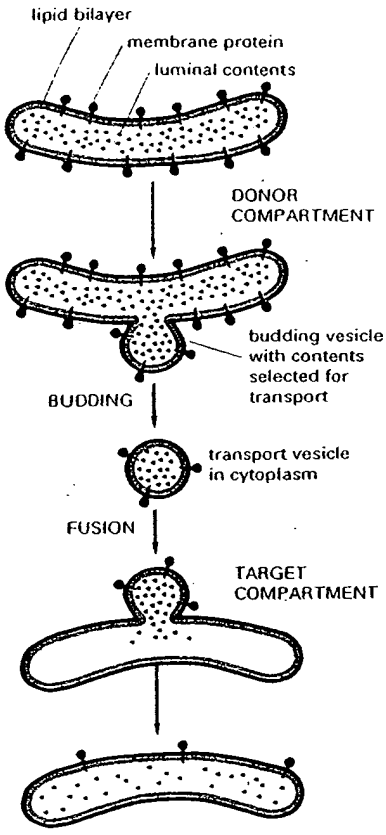


Figure 12–6 The "sidedness" of membranes is preserved during vesicular transport. Note that the original orientation of both proteins and lipids in the donor-compartment membrane is preserved in the target-compartment membrane and that soluble molecules are transferred from lumen to lumen.

through the membrane. The initial transport of selected proteins from the cytosol into the ER lumen or into mitochondria, for example, occurs in this way. (3) In **vesicular transport,** *transport vesicles* ferry proteins from one compartment to another. The vesicles become loaded with a cargo of molecules derived from the lumen of one compartment as they pinch off from its membrane; they discharge their cargo into a second compartment by fusing with its membrane. The transfer of soluble proteins from the ER to the Golgi apparatus, for example, occurs in this way. Because the transported proteins do not cross a membrane, they move only between compartments that are topologically equivalent (Figure 12–6). We discuss vesicular transport in more detail in Chapter 13. The three ways in which proteins are transported between different compartments are summarized in Figure 12–7.

Each of the three modes of protein transfer is usually selectively guided by sorting signals in the transported protein that are recognized by complementary receptor proteins in the target organelle. If a large protein is to be imported into the nucleus, for example, it must possess a sorting signal that is recognized by receptor proteins associated with the nuclear pore complex. If a protein is to be transferred directly across a membrane, it must possess a sorting signal that is recognized by the translocator in the membrane to be crossed. Likewise, if a protein is to be incorporated into certain types of transport vesicles or to be retained in certain organelles, its sorting signal must be recognized by a complementary receptor in the appropriate membrane.

Signal Peptides and Signal Patches Direct Proteins to the Correct Cellular Address [4]

There are at least two types of sorting signals on proteins. One type resides in a continuous stretch of amino acid sequence, typically 15 to 60 residues long. This **signal peptide** is often (but not always) removed from the finished protein by a specialized **signal peptidase** once the sorting process has been completed. The other type consists of a specific three-dimensional arrangement of atoms on the

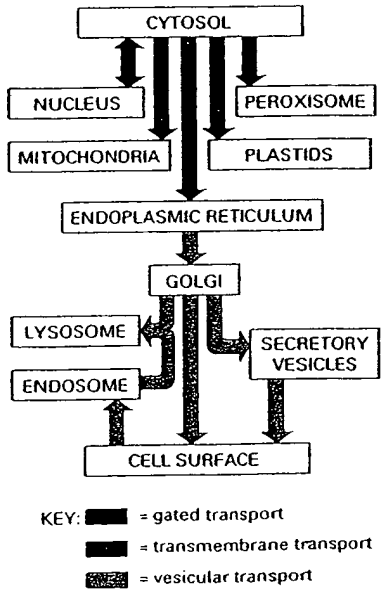


Figure 12–7 A simplified "road map" of protein traffic. Proteins can move from one compartment to another by gated transport (*red*), transmembrane transport (*blue*), or vesicular transport (*green*). The signals that direct a given protein's movement through the system, and thereby determine its eventual location in the cell, are contained in its amino acid sequence. The journey begins with the synthesis of a protein on a ribosome and terminates when the final destination is reached. At each intermediate station (*boxes*) a decision is made as to whether the protein is to be retained or transported further. In principle, a signal could be required either for retention in or for exit from each of the compartments shown, with the alternative fate being the *default pathway* (one that requires no signal). The vesicular transport of proteins from the ER through the Golgi apparatus to the cell surface, for example, appears not to require any specific sorting signals; specific sorting signals therefore are required to retain in the ER and the Golgi apparatus those specialized proteins that are resident there.

We shall use this figure repeatedly as a guide throughout this chapter and the next, highlighting the particular pathway being discussed.

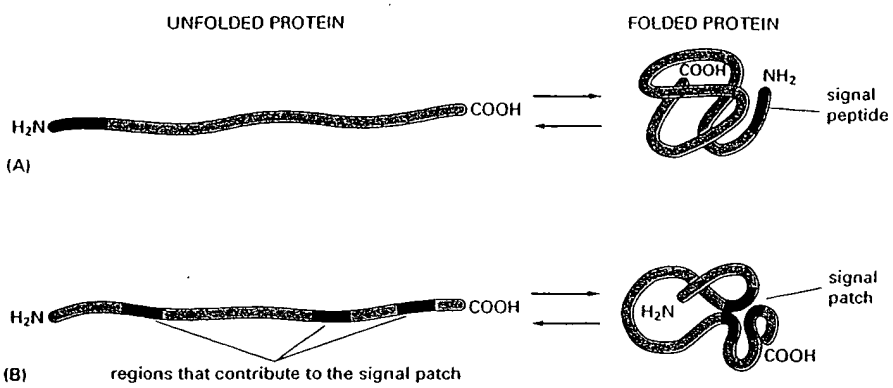


Figure 12–8 Two ways that a sorting signal can be built into a protein. (A) The signal resides in a single discrete stretch of amino acid sequence, called a *signal peptide*, that is exposed in the folded protein. Signal peptides often occur at the end of the polypeptide chain (as shown), but they can also be located elsewhere. (B) A *signal patch* can be formed by the juxtaposition of amino acids from regions that are physically separated before the protein folds (as shown); alternatively, separate patches on the surface of the folded protein that are spaced a fixed distance apart could form the signal. In either case the transport signal depends on the three-dimensional conformation of the protein, which makes it difficult to locate the signal precisely.

protein's surface that forms when the protein folds up. The amino acid residues that comprise this **signal patch** can be distant from one another in the linear amino acid sequence, and they generally remain in the finished protein (Figure 12–8). Signal peptides are used to direct proteins from the cytosol into the ER, mitochondria, chloroplasts, peroxisomes, and nucleus, and they are also used to retain soluble proteins in the ER. Signal patches identify certain enzymes that are to be marked with specific sugar residues that then direct them from the Golgi apparatus into lysosomes; signal patches are also used in other sorting steps that have been less well characterized.

Different types of signal peptides are used to specify different destinations in the cell. Proteins destined for initial transfer to the ER usually have a signal peptide at their amino terminus, which characteristically includes a sequence composed of about 5 to 10 hydrophobic amino acids. Most of these proteins will in turn pass from the ER to the Golgi apparatus, but those with a specific sequence of four amino acids at their carboxyl terminus are retained as permanent ER residents. Proteins destined for mitochondria have signal peptides of yet another type, in which positively charged amino acids alternate with hydrophobic ones. Proteins destined for peroxisomes usually have a specific signal sequence of three amino acids at their carboxyl terminus. Many proteins destined for the nucleus carry a signal peptide formed from a cluster of positively charged amino

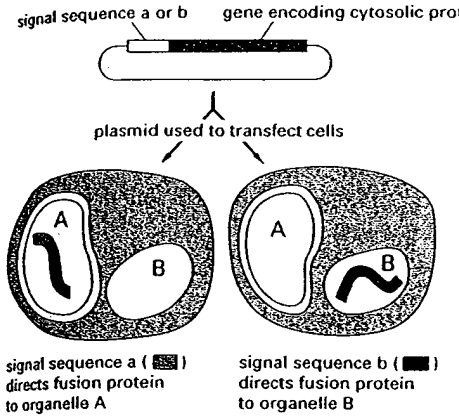
Table 12–3 Some Typical Signal Peptides

Function of Signal Peptide	Example of Signal Peptide
Import into ER	$^{+}H_3N$-Met-Met-Ser-Phe-Val-Ser- Leu-Leu-Leu-Val Gly-Ile-Leu-Phe-Trp-Ala -Thr-Glu-Ala-Glu- Gln-Leu-Thr-Lys-Cys-Glu-Val-Phe-Gln-
Retain in lumen of ER	-Lys-Asp-Glu-Leu-COO^{-}
Import into mitochondria	$^{+}H_3N$-Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe- Phe-Lys-Pro-Ala-Thr-Arg-Thr-Leu-Cys-Ser- Ser-Arg-Tyr-Leu-Leu-
Import into nucleus	-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-
Import into peroxisomes	-Ser-Lys-Leu-
Attach to membranes via the covalent linkage of a myristic acid to the amino terminus	$^{+}H_3N$-Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys-

Positively charged amino acids are shown in *red* and negatively charged amino acids in *green*. An extended block of hydrophobic amino acids is enclosed in a *yellow* box. H_3N^{+} indicates the amino terminus of a protein; COO^{-} indicates the carboxyl terminus.

A transfection approach for defining signal sequences

One way to show that a signal sequence is required and sufficient to target a protein to a specific intracellular compartment is to create a fusion protein in which the signal sequence is attached by genetic engineering techniques to a protein that is normally resident in the cytosol. After the cDNA encoding this protein is transfected into cells, the location of the fusion protein is determined by immunostaining or by cell fractionation.

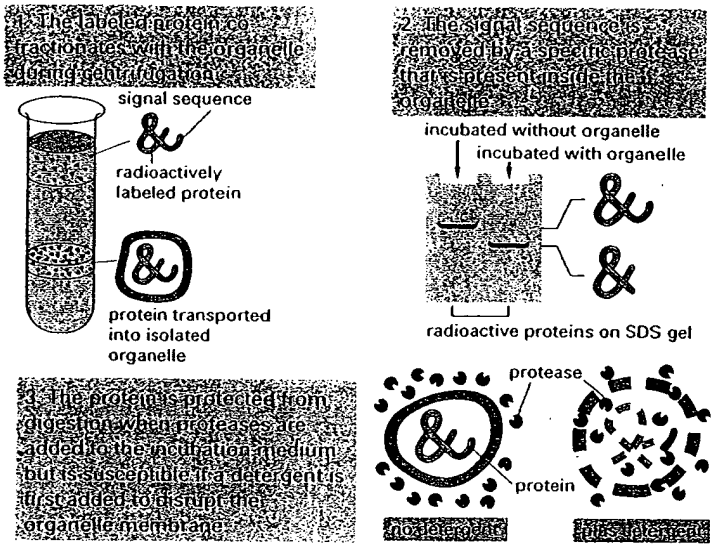


By altering the signal sequence using site-directed mutagenesis, one can determine which structural features are important for its function.

A biochemical approach for studying the mechanism of protein translocation

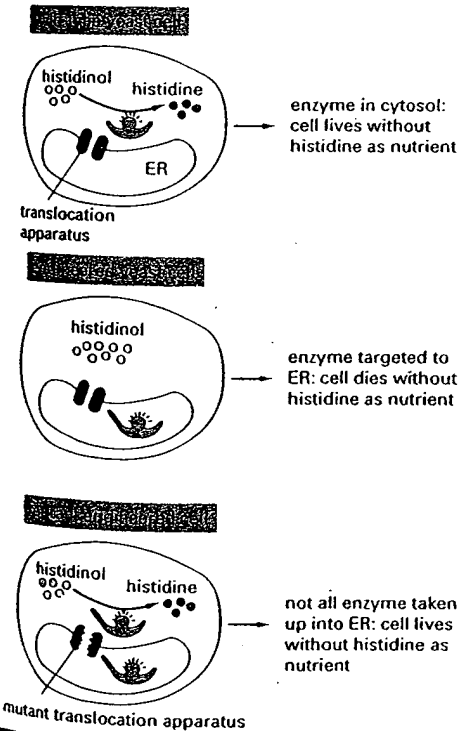
In this approach a labeled protein containing a specific signal sequence is transported into isolated organelles *in vitro*. The labeled protein is usually produced by cell-free translation of a purified mRNA encoding the protein; radioactive amino acids are used to label the newly synthesized protein so that it can be distinguished from the many other proteins that are present in the *in vitro* translation system.

Three methods are commonly used to test if the labeled protein has been translocated into the organelle:



By exploiting such *in vitro* assays, one can determine what components (proteins, ATP, GTP, etc.) are required for the translocation process.

Genetic approaches for studying the mechanism of protein translocation



Yeast cells with mutations in genes that encode components of the translocation machinery have been useful for studying protein translocation. Because mutant cells that cannot translocate proteins across their membranes will die, the trick is to design a strategy that allows weak mutations that cause only a partial defect in protein translocation to be isolated.

One way uses genetic engineering to design special yeast cells. The enzyme histidinol dehydrogenase, for example, normally resides in the cytosol, where it is required to produce the essential amino acid histidine from its precursor histidinol. A yeast strain is constructed in which the histidinol dehydrogenase gene is replaced by a re-engineered gene encoding a fusion protein with an added signal sequence that misdirects the enzyme into the endoplasmic reticulum (ER). When such cells are grown without histidine, they die because all of the histidinol dehydrogenase is sequestered in the ER, where it is of no use. Cells with a mutation that partially inactivates the mechanism for translocating proteins from the cytosol to the ER, however, will survive because enough of the dehydrogenase will be retained in the cytosol to produce histidine. Often one obtains a cell in which the mutant protein still functions partially at normal temperature but is completely inactive at higher temperature. A cell carrying such a temperature-sensitive mutation dies at higher temperature, whether or not histidine is present, as it cannot transport any protein into the ER. This allows the normal gene that was disabled by the mutation to be identified by transfecting the mutant cells with a yeast plasmid vector into which random yeast genomic DNA fragments have been cloned: the specific DNA fragment that rescues the mutant cells when they are grown at high temperature should encode the wild-type version of the mutant gene.

acids, which is commonly found at internal sites of the polypeptide chain. Some typical signal peptides are listed in Table 12–3.

The importance of each of these signal peptides for protein targeting has been shown by experiments in which the peptide is transferred from one protein to another by genetic engineering techniques: placing the amino-terminal ER signal peptide at the beginning of a cytosolic protein, for example, redirects the protein to the ER. Even though their amino acid sequences can vary greatly, the signal peptides of all proteins having the same destination are functionally interchangeable: physical properties, such as hydrophobicity, often appear to be more important in the signal-recognition process than the exact amino acid sequence.

Signal patches are far more difficult to analyze than signal peptides, and so less is known about their structure. Because they result from a complex three-dimensional protein-folding pattern, they cannot be easily transferred experimentally from one protein to another.

The main ways of studying how proteins are directed from the cytosol to a specific compartment and how they are translocated across membranes are illustrated in Panel 12–1 (p. 559).

Cells Cannot Construct Their Membrane-bounded Organelles *de Novo:* They Require Information in the Organelle Itself [5]

When a cell reproduces by division, it has to duplicate its membrane-bounded organelles. In general, cells do this by enlarging the existing organelles by incorporating new molecules into them; the enlarged organelles then divide and are distributed to the two daughter cells. Thus each daughter cell inherits from its mother a complete set of specialized cell membranes. This inheritance is essential because a cell could not make such membranes *de novo.* If the ER were completely removed from a cell, for example, how could the cell reconstruct it? The membrane proteins that define the ER and carry out many of its functions are themselves products of the ER. A new ER could not be made without an existing ER or, at the very least, a membrane that contains the translocators required to import specific proteins into the ER (and lacks the translocators required to import the proteins that function in other organelles).

Thus it seems that the information required to construct a membrane-bounded organelle does not reside exclusively in the DNA that specifies the organelle's proteins. *Epigenetic* information in the form of at least one distinct protein that preexists in the organelle membrane is also required, and this information is passed from parent cell to progeny cell in the form of the organelle itself. Presumably, such information is essential for the propagation of the cell's compartmental organization, just as the information in DNA is essential for the propagation of its nucleotide and amino acid sequences.

Summary

Eucaryotic cells contain intracellular membranes that enclose nearly half the cell's total volume in separate intracellular compartments called organelles. The main types of membrane-bounded organelles that are present in all eucaryotic cells are the endoplasmic reticulum, Golgi apparatus, nucleus, mitochondria, lysosomes, endosomes, and peroxisomes; plant cells also contain plastids, such as chloroplasts. Each organelle contains a distinct set of proteins that mediates its unique functions.

Each newly synthesized organelle protein finds its way from the ribosome where it is made to the organelle where it functions by following a specific pathway, guided by signals in its amino acid sequence that function as signal peptides or signal patches. The signal peptides and patches are recognized by complementary receptor proteins in the target organelle. Proteins that function in the cytosol do not contain signal peptides or signal patches and therefore remain in the cytosol after they are synthesized.

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Signal peptide cleavage of a type I membrane protein, HCMV US11, is dependent on its membrane anchor

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The human cytomegalovirus (HCMV) US11 polypeptide is a type I membrane glycoprotein that targets major histocompatibility complex (MHC) class I molecules for destruction in a proteasome-dependent manner. Although the US11 signal sequence appears to be a classical N-terminal signal peptide in terms of its sequence and cleavage site, a fraction of newly synthesized US11 molecules retain the signal peptide after the N-linked glycan has been attached and translation of the US11 polypeptide has been completed. Delayed cleavage of the US11 signal peptide is determined by the first four residues, the so-called n-region of the signal peptide. Its replacement with the four N-terminal residues of the H-2K^b signal sequence eliminates delayed cleavage. Surprisingly, a second region that affects the rate and extent of signal peptide cleavage is the transmembrane region close to the C-terminus of US11. Deletion of the transmembrane region of US11 (US11-180) significantly delays processing, a delay overcome by replacement with the H-2K^b signal sequence. Thus, elements at a considerable distance from the signal sequence affect its cleavage.

Keywords: ER subdomains/HCMV US11/post-translational ER processing/signal sequence cleavage/transmembrane anchor

Introduction

Membrane proteins and proteins destined for secretion are targeted to the appropriate intracellular membrane by their signal peptides (Martoglio and Dobberstein, 1998). In eukaryotes, signal peptides are 15–50 amino acids long and are usually located at the N-terminus (von Heijne, 1983). A typical signal peptide is comprised of three distinct regions: a polar N-terminal end (n-region) that may have a net positive charge, a central hydrophobic core (h-region) that consists of 6–15 hydrophobic amino acids, and a polar C-terminal (c-region) end that contains prolines and glycines (von Heijne, 1985). A signal peptide containing the consensus sequence and proper cleavage site ensures that proteins are inserted into the endoplasmic reticulum (ER) membrane and are processed properly. Mutations within the sequence immediately downstream of the signal peptide affect protein processing, and can result in both inefficient and inaccurate cleavage (Russel and Model, 1981; Folz and Gordon, 1986; Andrews *et al.*, 1988; Wiren *et al.*, 1988). For example, replacement of glutamic acid for leucine at the +2 position of the phage coat protein cleavage site causes inefficient removal of its signal peptide (Russel and Model, 1981). When the propeptides of human pre-pro-apolipoprotein A-II and pre-pro-parathyroid hormone are deleted, five and six residues, respectively, the generation of an improper N-terminus and a failure to direct the nascent chain to the ER properly are observed (Folz and Gordon, 1986; Wiren *et al.*, 1988). Elements of the nascent chain at greater distances from the signal peptide are not known to affect signal peptide processing.

Shortly after its translation, the signal peptide interacts with signal recognition particle (SRP) and causes translational arrest (Walter and Blobel, 1981; Walter and Johnson, 1994). SRP is a ribonucleoprotein comprised of a 7S RNA associated with six different polypeptides (Walter and Blobel, 1980, 1982). The 54 kDa subunit of SRP interacts with the signal peptide through a hydrophobic region that promiscuously accommodates signal peptides of different lengths and sequences (Keenan *et al.*, 1998). The SRP–nascent polypeptide chain–ribosome complex is targeted to the ER membrane where SRP binds to the SRP receptor and the ribosome weakly interacts with the translocon (mainly comprised of the Sec61p complex) (Gorlich *et al.*, 1992; Kalies *et al.*, 1994; for reviews see Rapoport *et al.*, 1996; Hegde and Lingappa, 1999; Johnson and van Waes, 1999). The signal peptide is then transferred from the SRP into the channel of the translocon, where it directly associates with the Sec61α subunit of the Sec61 complex to promote tight interaction of the ribosome–nascent chain complex with the translocon (Jungnickel and Rapoport, 1995; Mothes *et al.*, 1998; Plath *et al.*, 1998). The signal peptide can also associate with the lipid bilayer and the TRAM protein (Martoglio *et al.*, 1995; Voigt *et al.*, 1996; Mothes *et al.*, 1997), which assists in protein transport through the translocon. The interaction of the signal peptide with the Sec61 complex may also induce the removal of a 'gating factor', possibly BiP, from the lumenal side of the translocon, to allow access of the nascent polypeptide to the ER lumen (Crowley *et al.*, 1994; Hamman *et al.*, 1998). Chain elongation is re-initiated, followed by signal peptide translocation through the Sec61 channel. The hydrophobic nature of the signal peptide allows its insertion into the ER membrane, followed by signal peptidase cleavage upon lumenal exposure of the cleavage site (Blobel and Dobberstein, 1975). This cleavage site is characterized by small uncharged residues at positions –1 and –3 (von Heijne, 1990). After signal peptide cleavage,

chain elongation of the nascent chain continues, while the signal peptide itself can be cleaved further by aminopeptidases or signal peptide peptidase (Lyko *et al.*, 1995; Martoglio *et al.*, 1997).

Signal peptidase is an endopeptidase that resembles other serine proteases (Dalbey and von Heijne, 1992) and performs a similar cleavage reaction for prokaryotic and eukaryotic signal peptidases. The crystal structure of the periplasmic domain of *Escherichia coli* leader peptidase (Paetzel *et al.*, 1998) reveals important mechanistic aspects of signal peptide cleavage: the catalytic site proposed to be close to the lipid bilayer is surrounded by a hydrophobic region, explaining the requirement for small uncharged, aliphatic residues at the −1 and −3 positions of the cleavage site (Paetzel *et al.*, 1998; von Heijne, 1998). The mammalian signal peptidase complex (SPC) is comprised of at least five subunits with molecular masses of 25, 23/22, 21, 18 and 12 kDa (Evans *et al.*, 1986). The non-catalytic subunits of the eukaryotic SPC may function as regulatory subunits for signal peptide recognition and are located in close proximity to the translocon (Meyer and Hartmann, 1997). The Sec61p complex interacts with the 25 kDa subunit of the SPC (SPC25), which suggests a tight interaction between the SPC and the Sec61 complex (Kalies *et al.*, 1998). This interaction may serve to recruit the SPC to the translocation site and thereby enhance the overall translocation efficiency of the nascent polypeptide.

The human cytomegalovirus (HCMV) gene products US11 and US2 target the major histocompatibility complex (MHC) class I molecules for destruction by the proteasome (Wiertz *et al.*, 1996a,b; Tortorella *et al.*, 1998). These viral proteins associate with the class I molecules in the ER and induce the dislocation of the class I heavy chains from the ER, probably via the Sec61p complex, for degradation in the cytosol (Wiertz *et al.*, 1996b). In all likelihood, a similar set of reactions is utilized for the removal and degradation of misfolded and abnormal ER proteins more generally (Bonifacino and Weissman, 1998). The HCMV US11 gene product is an ER-resident type I membrane glycoprotein (Figure 1), the single N-linked glycan attachment site of which is glycosylated quantitatively. The hydrophobic stretch at the N-terminus of US11 is characteristic of a signal peptide, while the hydrophobic stretch at the C-terminal end corresponds to a transmembrane/stop transfer sequence.

Here we report a highly unusual cleavage pattern for the US11 signal peptide. At least a fraction of the US11 signal peptide appears to be cleaved post-translationally. This trait is determined by the US11 signal peptide n-region. What cleavage occurs is also strongly influenced by the US11 transmembrane domain. Delayed cleavage of the US11 signal peptide may reflect the local ER environment in which dislocation takes place.

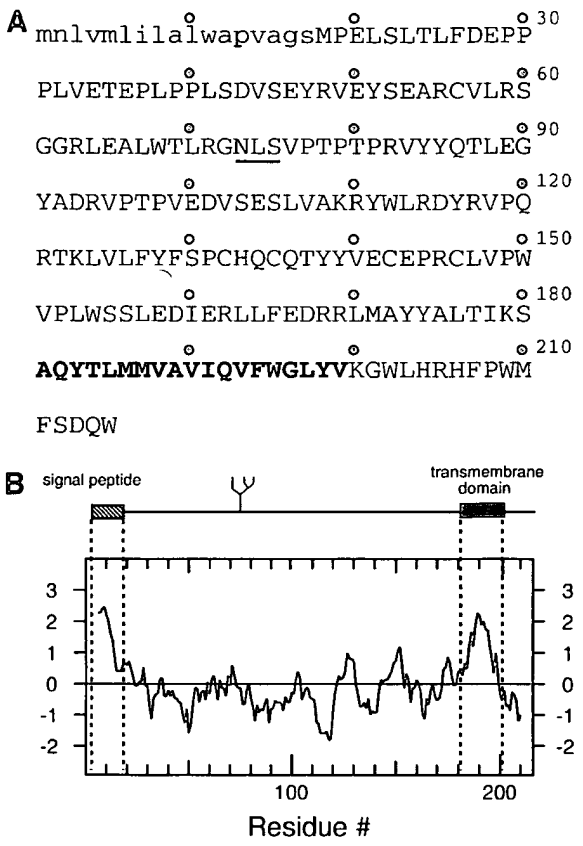


Fig. 1. (**A**) Amino acid sequence (single letter code) of HCMV US11. (**B**) Kyte–Doolittle hydropathy plot of US11. The predicted signal sequence is depicted in lower case. Bold face type represents the predicted transmembrane domain. The N-linked glycosylation site is underlined.

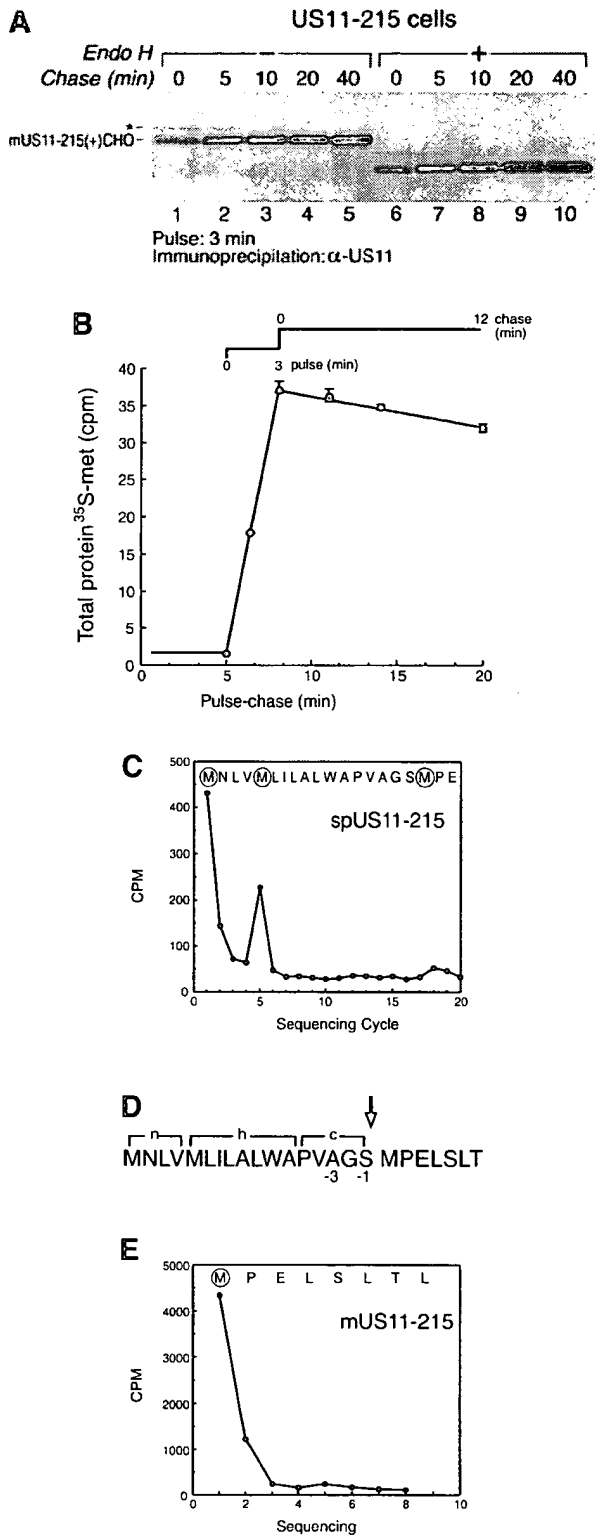
Results

The HCMV US11 signal peptide is cleaved post-translationally

HCMV US11 is a 215 residue ER-resident protein that targets MHC class I heavy chains for destruction by the proteasome. The detailed mechanism by which the viral gene product accomplishes this is unclear, but is closely coupled to the biosynthesis of the class I and US11 products. We therefore examined whether the biosynthesis of US11 might reveal unique properties of the ER environment in which US11 normally functions. The maturation of US11 was examined in U373-MG cells stably transfected with US11 (US11-215 cells). US11-215 cells were metabolically labeled for 3 min with [^{35}S]methionine and chased for up to 40 min. The US11 protein was recovered from cell lysates by immunoprecipitation using a polyclonal anti-US11 serum (α-US11) and analyzed by SDS–PAGE (Figure 2A). Two species of US11 of distinct mobility were recovered at early time points (Figure 2B, lanes 1 and 2). The faster moving, major species is the ER-resident, mature form of US11 (mUS11-215). It has a mobility indistinguishable from that of US11 recovered from a microsome-supplemented cell-free translation system (D.Tortorella and H.L.Ploegh, unpublished data).

A precursor–product relationship between the two species was suggested by increased recovery at later chase points of mUS11-215 and decreased recovery of the slower moving species (*) (Figure 2A, lanes 1–4). The identity of the slower moving species (*) was unclear. Is it a distinct form of US11 or is it a protein associated with US11? Both mUS11-215 and the slower moving polypeptide (*) were recovered from SDS-denatured primary

immunoprecipitates in a second round of immunoprecipitation using α-US11 serum (D.Tortorella and H.L.Ploegh, unpublished data). We therefore conclude that the slowly migrating polypeptide is a distinct form of the US11 protein.



The precursor–product conversion observed for the slower moving polypeptide (*) and mUS11-215 does not account fully for the amount of US11 recovered at early chase times. At the early time points of chase, there is a shortfall in the recovery of US11 (Figure 2A, lanes 1–3). This shortfall is not due to the continued incorporation of label during the chase (Figure 2B) and hence must result from the inability to retrieve all US11 at the early time points. Solubilization with the detergent SDS significantly improved recovery of both US11 polypeptides (* and mUS11-215) at the early time points (D.Tortorella and H.L.Ploegh, unpublished data).

Earlier experiments failed to show the presence of endoglycosidase H (Endo H)-resistant US11 and indicated that US11 was confined to the ER, as confirmed by immunoelectron microscopy (Wiertz *et al.*, 1996a). The primary structure of US11 predicts a single N-linked glycan (CHO) attachment site at position 73 (Asn73–Leu–Ser) (Figure 1). Both polypeptides (* and mUS11-215) recovered from the US11 immunoprecipitates were susceptible to digestion by Endo H (Figure 2A, lanes 6–10). The difference between these two molecules of US11 cannot be due to an unusual modification of the N-linked glycan and, therefore, must be caused by differences in the polypeptide backbone.

What type of modification could account for the presence of the slower moving species of US11? Based on the observed apparent molecular weight, the slowly migrating species of US11 may still contain the N-terminal signal peptide (spUS11-215). The polypeptide was isolated from [^{35}S]methionine-labeled cells and subjected to 20 cycles of Edman degradation (Figure 2C). The observed peaks of radioactivity fit the position of the methionines at the N-terminal end of the US11 precursor sequence. These results establish that, surprisingly, the slower moving form (*) (Figure 2A) is indeed a glycosylated US11 molecule that has retained its signal peptide.

The US11 signal peptide contains a typical cleavage site

The factor known to influence signal peptide cleavage is the presence of small amino acid side chains at the −1 and −3 position relative to the cleavage site. Does the US11 signal peptide cleavage site indeed contain the consensus

Fig. 2. Two forms of US11 exist early in biosynthesis. (**A**) US11-215 cells were pulsed for 3 min and chased for up to 40 min. Cells were lysed in 0.5% NP-40 and immunoprecipitated with anti-US11 serum (α-US11). The precipitates were analyzed by SDS–PAGE (12.5%). Two forms of US11 [* and mature US11-215 (+)CHO] were recovered from the US11-215 cell lysates (lanes 1–5). Half of the α-US11 precipitates were digested with Endo H (lanes 6–10). (**B**) Incorporation of [^{35}S]methionine was examined during a pulse–chase experiment of US11-215 cells. TCA-precipitable radioactivity (c.p.m.) from [^{35}S]methionine of each time point was plotted against the pulse–chase experiment. An average of three samples is represented at each value. (**C**) The slower moving US11 polypeptide (*) was subjected to N-terminal radiosequencing. The radioactivity (c.p.m.) from [^{35}S]methionine of each fraction of the N-terminal radiosequencing run was plotted against Edman cycle number. (**D**) The n-, h- and c-regions of the US11 signal peptide are shown. The site of signal peptide cleavage is indicated by an arrow. (**E**) N-terminal radiosequencing of the mature form of US11 (mUS11-215) plotted as radioactivity (c.p.m.) from [^{35}S]methionine versus Edman cycle number.

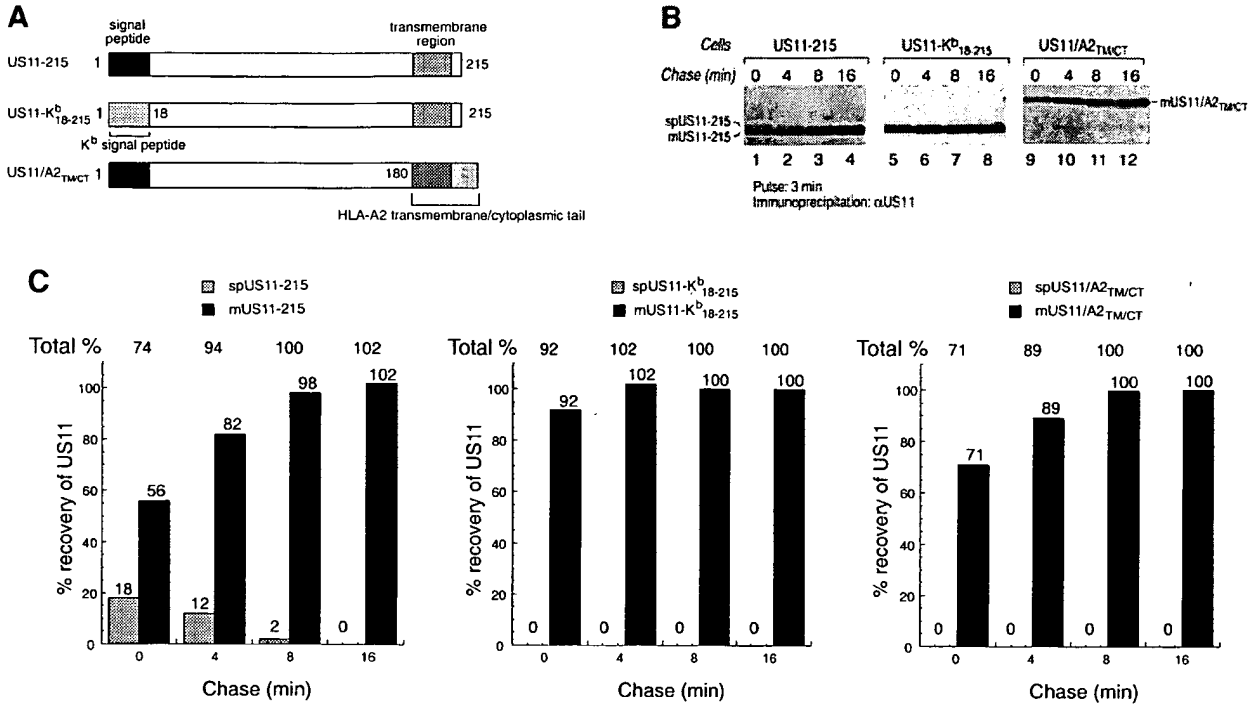


Fig. 3. The delayed cleavage of the US11 signal peptide is determined by its signal sequence and transmembrane/cytoplasmic tail region. (**A**) The US11 chimeric molecules US11–K^b_{18-215}, US11/$A2_{TM/CT}$ and wild-type US11-215. (**B**) Processing of these molecules was examined in stable transfectants of MG-U373 cells using pulse–chase analysis. US11 was recovered from SDS lysates using α-US11 serum and analyzed by SDS–PAGE (12.5%). The signal peptide-containing form of US11 (spUS11-215) and the mature processed form of US11 (mUS11-215) are indicated. (**C**) The US11-215 molecules recovered from (B) were quantitated by a Molecular Dynamics Storm PhosphorImager. The US11 recovered at each time point is represented as percentage recovery of US11. The US11 recovered at the 8 min chase point was used as the 100% recovery value.

amino acids at the proper position? Analysis of the US11 primary sequence using the SignalP program (www.cbs.dtu.dk/services/SignalP/index.html) (Nielsen *et al.*, 1997a,b) predicts signal peptide cleavage of US11 to occur between residues 17 and 18 (Figure 2D). Serine (17) occurs at position –1 and alanine (15) at position –3, residues that are in perfect agreement with the consensus sequence for a signal peptide cleavage site. Methionine would be the N-terminus of the processed US11 molecule. Indeed, US11 isolated from [^{35}S]methionine-labeled US11-215 cells and subjected to eight cycles of N-terminal sequencing (Edman degradation) yielded methionine at position 1 (Figure 2E). Methionines within the N-terminal sequence of US11 occur at positions 5 and 18. Removal of only four residues from the N-terminus would not account for the mobility difference between the two forms of US11. Therefore, the methionine at position 18 must be the first residue of the mature US11 molecule. These results suggest that the unusual cleavage pattern of the US11 signal peptide is not due to an anomalous signal peptidase cleavage site.

The US11 signal peptide and the transmembrane region contribute to the delayed cleavage of the US11 signal sequence

N-terminal signal peptide cleavage is presumably determined solely by the sequence of the signal peptide itself (Martoglio and Dobberstein, 1998). Changes within the n-, h- or c-region of the signal peptide and the regions directly downstream from the signal peptide affect signal peptide processing (Russel and Model, 1981; Folz and Gordon, 1986; Wiren *et al.*, 1988; Izard and Kendall, 1994). Can the US11 signal peptide itself or regions further downstream of the US11 signal sequence, such as the US11 transmembrane region, play a role in signal peptide cleavage? We generated US11–K^b_{18-215} (Figure 3A), a chimeric molecule in which the US11 signal peptide was replaced with the signal peptide of the murine MHC class I heavy chain H-2K^b, a type I membrane protein. We also generated US11/$A2_{TM/CT}$ (Figure 3A), a chimeric molecule in which the transmembrane and cytoplasmic tail of US11 were replaced with the corresponding regions of human MHC class I heavy chain A2. Cleavage of the H-2K^b signal peptide should now generate the N-terminus of mature US11. Pulse–chase analysis of US11-215 cells shows the recovery of spUS11-215 and mUS11 at the early times points and a precursor–product relationship between the two polypeptides (Figure 3B, lanes 1–4, and C). For neither US11–K^b_{18-215} nor US11/$A2_{TM/CT}$ did we observe the presence of a signal sequence-containing precursor (Figure 3B, lanes 5–8 and 9–12). This result suggests that unique features of US11's signal sequence and transmembrane domain contribute to the persistence of spUS11-215.

The recovery of mUS11-215 and US11/$A2_{TM/CT}$ increases with time (Figure 3B, lanes 1–4 and 9–12, and C). In contrast, recovery of US11–K^b_{18-215} does not significantly change during the chase (Figure 3B, lanes 5–8, and C). We therefore conclude that the US11 signal

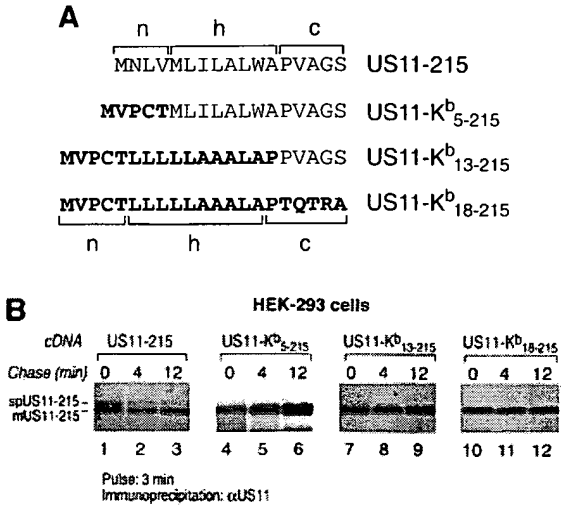


Fig. 4. The n-region of the US11-215 signal peptide is responsible for its delayed cleavage. (**A**) The amino acid sequences of the n-, h- and c-regions of US11-215, US11–K^b_{5-215}, US11–K^b_{13-215} and US11–K^b_{18-215}. Bold letters represent the H-2K^b signal peptide. (**B**) US11–K^b_{5-215} (lanes 4–6), US11–K^b_{13-215} (lanes 7–9) and US11–K^b_{18-215} (lanes 10–12) were transfected in HEK-293 cells and analyzed by pulse–chase analysis. US11 was recovered from SDS lysates using α-US11 serum and analyzed by SDS–PAGE (12.5%). The signal peptide-containing form of US11 (spUS11-215) and the mature processed form of US11 (mUS11-215) are indicated.

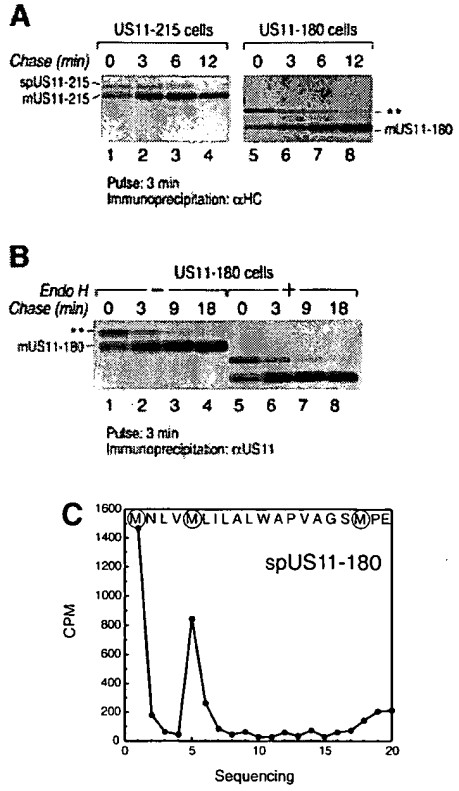


Fig. 5. Signal peptide cleavage of US11-180 is significantly delayed. (**A**) Processing of US11-215 and US11-180 was examined in stable transfectants of MG-U373 cells using pulse–chase analysis. US11 was recovered from SDS lysates using α-US11 serum and analyzed by SDS–PAGE (12.5%). The signal peptide-containing form of US11 (spUS11-215) and the mature processed form of US11 (mUS11-215) were immunoprecipitated from US11-215 cells (lanes 1–4). Two major species, ** and the mature processed form of US11-180 (mUS11-180), were recovered from US11-180 cells. (**B**) Half of the α-US11 precipitate recovered from a pulse–chase experiment of US11-180 cells was digested with Endo H (lanes 5–8). (**C**) The slower moving US11-180 polypeptide (**) was subjected to N-terminal radiosequencing. The radioactivity (c.p.m.) recovered at each Edman cycle is shown.

peptide is also responsible for the increased recovery of US11-215 and US11/A2$_{TM/CT}$ at the later time points. We suggest that the manner in which the US11 signal peptide initiates contact with the ER may contribute to its solubility properties.

The n-, h- and c-regions of the US11 signal peptide follow the proposed consensus for a cleavable N-terminal signal peptide. However, the results obtained for the chimeric US11–K^b_{18-215} molecule suggest that the signal peptide itself may account for its delayed cleavage. To characterize further the segment of the US11 signal peptide that is responsible for delayed cleavage, we generated additional chimeras in which the n-region (US11–K^b_{5-215}) or n + h-regions (US11–K^b_{13-215}) of US11 are replaced with the corresponding regions of H-2K^b (Figure 4A). We transfected US11-215, US11–K^b_{5-215}, US11–K^b_{13-215} and US11–K^b_{18-215} into HEK-293 cells and examined their processing by pulse–chase analysis (Figure 4B). For US11-215, a signal peptide-containing form of US11 and the mature form of US11-215 were evident at early chase times (Figure 4B, lanes 1–3). The two polypeptides showed a precursor–product relationship. For the chimeras US11–K^b_{5-215}, US11–K^b_{13-215} and US11–K^b_{18-215}, removal of the signal peptide is rapid and only the mature, cleaved form of US11 is recovered (Figure 4B, lanes 4–12). We conclude that features within the n-region of the US11 signal peptide contribute to its persistence.

During the chase, there is an increase in recovery of the mature form of US11–K^b_{5-215} and US11–K^b_{13-215} (Figure 4B, lanes 4–9), but not for US11–K^b_{18-215} (Figures 4B, lanes 10–12, and 3B, lanes 5–8, and C). Therefore, the c-region of the US11 signal peptide somehow contributes to recovery of mature US11. While the identity of the c-region does not affect the cleavage of the signal peptide, it does contribute to the recovery of mature US11. Perhaps the c-region is responsible for positioning nascent US11 relative to other components of the translocation machinery. This positioning may affect interactions of US11 with other ER components shortly after its completion, and hence its solubility. In contrast, the presence of the full K^b signal sequence neither delays signal peptide cleavage nor affects the recovery of US11 from cell lysates.

The US11 transmembrane region plays a role in US11 signal peptide cleavage

We next examined the role of the US11 transmembrane region in signal peptide cleavage. Such a role was suggested by the analysis of the US11/A2$_{TM/CT}$ chimeric construct (Figure 3). We generated a C-terminal truncation of US11 that lacks the predicted transmembrane segment and the cytoplasmic tail (US11-180) (Figure 1). The

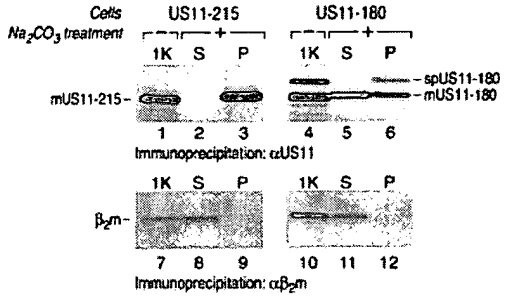


Fig. 6. US11-180 is a soluble molecule. US11-215 and US11-180 cells were metabolically labeled for 15 min. The cells were homogenized with glass beads and centrifuged at 1000 *g*. The 1000 *g* supernatant fractions were treated with 100 mM Na_2CO_3, followed by centrifugation at 150 000 *g*. US11 molecules (lanes 1–6) and β_2m (lanes 7–12) were recovered from the 1000 *g* (1K) pellet, 150 000 *g* supernatant (S) and the 150 000 *g* pellet (P) using α-US11 and α-β_2m serum. The immunoprecipitates were analyzed by SDS–PAGE (12.5%).

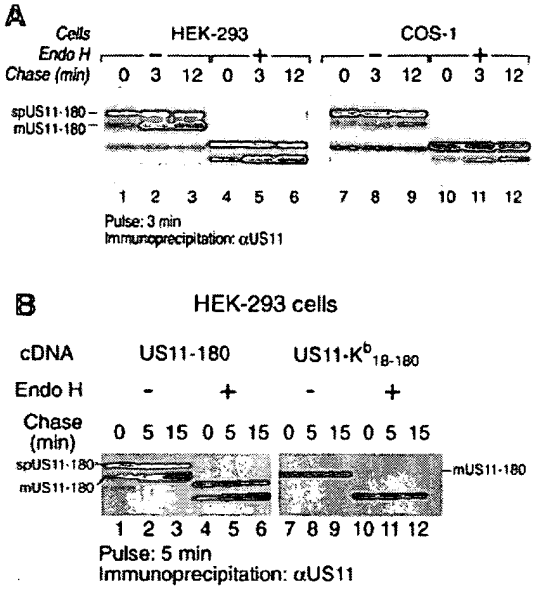


Fig. 7. The US11 signal peptide plays a major role in processing of US11-180. US11-180 cDNA was transfected into HEK-293 and COS-1 cells. (**A**) Processing of US11-180 was examined by pulse–chase analysis. US11-180 was recovered from SDS lysates using α-US11 serum and analyzed by SDS–PAGE (12.5%). Half of the immunoprecipitates recovered from the respective transfectants were treated with Endo H (lanes 4–6 and 10–12). (**B**) The US11 signal peptide chimeric molecule US11–K$^b_{18-180}$ and US11-180 were transfected in HEK-293 cells and analyzed by pulse–chase analysis. US11 was recovered from SDS lysates using α-US11 serum and analyzed by SDS–PAGE (12.5%). Half of the immunoprecipitates recovered from the respective transfectants were treated with Endo H (lanes 4–6 and 10–12). The signal peptide-containing form of US11 (spUS11-180) and the mature processed form of US11 (mUS11-180) are indicated.

processing of wild-type US11-215 and US11-180 was examined in the appropriate U373-MG transfectants (Figure 5A). US11 recovered at the early chase times from US11-215 cells produced the usual pattern with respect to the precursor–product relationship of spUS11-215 and mUS11-215 (Figure 5A, lanes 1–4). Two major species were recovered from US11-180 cells (** and mUS11-180) (Figure 5A, lanes 5–8). A precursor–product relationship exists for the slower (**) and faster migrating species (mUS11-180) of US11-180. The two polypeptides recovered from the US11-180 transfectants represent distinct forms of the polypeptide backbone and both species of US11-180 are sensitive to Endo H (Figure 5B, compare lanes 1–4 and 5–8).

The slower moving species (**) was isolated from a US11-180 HEK-293 transfectant labeled with [^{35}S]methionine and subjected to 20 cycles of Edman degradation (Figure 5C). The data showed persistence of the signal sequence. The absence of the transmembrane region of US11 thus strongly delays cleavage of its N-terminal signal peptide. An even more pronounced result was observed when US11-180 cDNA was transfected into HEK-293 and COS-1 cells (Figure 7).

mUS11-180 is a soluble protein

The Kyte–Doolittle hydropathy plot of US11 (Figure 1) suggests that the transmembrane region is located between residues 180 and 200. However, the hydrophobic nature of residues 180–200 does not ensure that it is in fact a transmembrane anchor. All attempts at proteolytic removal of the proposed cytoplasmic tail were without success. We performed Na_2CO_3 extractions to explore stable membrane insertion of US11-215 and US11-180 (Figure 6). US11-215 and US11-180 cells were labeled with [^{35}S]methionine and broken with glass beads in the absence of detergent. Homogenates were then centrifuged at 1000 *g* to remove large debris, and the supernatant fraction was treated with 100 mM Na_2CO_3, followed by centrifugation at 150 000 *g* to sediment the extracted microsomes. US11-215 and US11-180 molecules were immunoprecipitated from detergent extracts prepared from the 1000 *g* pellet (Figure 6, lanes 1 and 4), the Na_2CO_3-treated 150 000 *g* soluble fraction (Figure 6, lanes 2 and 5) and the 150 000 *g* pellet fraction (Figure 6, lanes 3 and 6). As a soluble, lumenal control protein, we used β_2-microglobulin (β_2m) (Figure 6, lanes 7–12). The US11-215 polypeptide is recovered exclusively from the 150 000 *g* pellet fraction (Figure 6, lane 3), whereas the bulk of β_2m is recovered from the 150 000 *g* soluble fraction (Figure 6, lane 8). These results confirm that US11-215 is a membrane protein. In contrast, the majority of US11-180 lacking its signal peptide (mUS11-180) and β_2m are recovered from the 150 000 *g* soluble fraction (Figure 6, lanes 5 and 11). These results confirm that mUS11-180 and β_2m are soluble, ER lumenal proteins.

A small fraction of mUS11-180 is recovered from the 150 000 *g* pellet fraction (Figure 6, lane 6) and may represent mUS11-180 that continues to associate with the ER membrane shortly after signal peptide cleavage and prior to its release into the ER lumen. Alternatively, a fraction of mUS11-180 may interact with an ER membrane protein in a Na_2CO_3-resistant manner. As might be expected, the signal peptide-containing form of US11-180 (spUS11-180) remains associated with the membrane fraction even after carbonate extraction (Figure 6, lane 6).

The identity of the signal sequence dictates delayed cleavage of the US11-180 molecule

For reasons of consistency with the data shown earlier, the experiments in Figure 5A were all conducted in U373-MG

cells stably transfected with the US11-180 cDNA. The delayed cleavage of the signal peptide of US11 is not an aberration of the recipient cell line used for transfection. In fact, when we used either HEK-293 or COS-1 cells in a transient transfection protocol, the persistence of the signal peptide-containing form of both US11-215 (Figure 4B, lanes 1–3) and US11-180 (Figure 7A) was much more pronounced. The relative amount of signal sequence-containing precursor of US11-180 was increased to the extreme, such that in COS-1 cells it is in fact the predominant form of US11-180 at the end of the chase (Figure 7A, lanes 7–12). Our data show that the anomalous behavior of the US11 signal peptide is intrinsic to the US11 molecule. In transfection experiments exploiting COS-1 cells to express other type I membrane proteins, the persistence of signal peptides was not observed (Huppa and Ploegh, 1997) and to our knowledge has not been reported by others.

We next addressed the contribution of the signal sequence's identity to the delayed cleavage observed for US11-180. We generated a chimeric molecule, US11–K^b_{18-180}, in which the US11-180 signal peptide is replaced with the H-$2K^b$ signal peptide (Figure 3A). We transfected US11-180 and US11–K^b_{18-180} into HEK-293 cells and examined their processing by pulse–chase analysis (Figure 7B). The immunoprecipitates were treated with Endo H to verify glycosylation and ER insertion (Figure 7B, lanes 4–6 and 10–12). For US11-180 carrying the US11 signal peptide, the signal peptide-containing form of spUS11-180 and the mature processed form of US11-180 were observed throughout the chase (Figure 7B, lanes 1–3). In contrast, a single polypeptide with a mobility similar to that of mUS11-180 is recovered from US11–K^b_{18-180} transfectants (Figure 7B, lanes 7–9). Delayed cleavage of the US11-180 signal peptide no longer occurs when the US11 signal peptide is replaced with the H-$2K^b$ signal peptide. Not only the US11 transmembrane segment, but also features of the US11 signal sequence itself play a major role in US11 signal peptide cleavage.

Discussion

We describe here the unusual properties of the signal sequence of HCMV US11, a type I membrane glycoprotein. Elements contained within the signal sequence's N-terminal segment (Met–Asn–Leu–Val) are responsible for delayed cleavage, such that a fully glycosylated, signal peptide-bearing intermediate is readily detected. In addition, the C-terminal membrane anchor also affects the rate of signal peptide cleavage; a US11 variant lacking its transmembrane/cytoplasmic tail segment (US11-180) shows an even greater delay in signal peptide cleavage than is seen for full-length US11. This effect is at its most extreme in COS-1 cells, where the glycosylated, signal peptide-containing US11-180 protein (spUS11-180) is the majority of US11 polypeptide that persists. To account for these findings, we propose an extended interaction of the signal peptide and transmembrane segment with the processing apparatus.

Conformity with the consensus parameters within the n-, h- and c-regions of the signal peptide predicts proper cleavage of an N-terminal signal peptide. The US11 signal peptide sequence fits the consensus parameters within the n-, h- and c-regions, yet fails to be cleaved efficiently from the nascent chain. Chimeric molecules in which regions (n, n + h or n + h + c) of the US11 signal peptide were replaced with the corresponding regions of the murine class I heavy chain H-$2K^b$ signal peptide demonstrate that it is the n-region of the US11 signal sequence that is mostly responsible for the delayed cleavage of the US11 signal peptide (Figure 4). An irregular n-region has been observed to affect signal peptide processing; a surfeit of positive charges within the n-region of the HIV-1 gp-120 signal sequence probably accounts for its inefficient cleavage (Li *et al.*, 1994, 1996). This aberrant form of gp-120 does not exit the ER and, therefore, cannot be incorporated into a nascent virion. We note that the persistence of the uncleaved signal sequence on gp-120 was never directly shown by sequence analysis.

Regions outside the signal peptide can also influence its cleavage. In pre-pro-apolipoprotein A-II and pre-pro-parathyroid hormone, removal of the propeptide that is immediately downstream of the signal peptide influenced ER protein translocation and proper signal peptide processing (Russel and Model, 1981; Folz and Gordon, 1986; Andrews *et al.*, 1988; Wiren *et al.*, 1988). These changes mostly affect the site of cleavage, shifting it a few residues downstream, while their effect on the rate of signal peptide cleavage was not addressed in any detail. In addition, a mutation at the +2 position of the signal peptide cleavage site of phage coat protein also results in inefficient cleavage (Russel and Model, 1981). All of these mutations are localized immediately downstream of the signal peptide. In contradistinction to such signal sequence-proximal alterations, the transmembrane anchor of US11, at a considerable distance (~160 residues) from the US11 signal sequence, strongly influences signal sequence cleavage. The rate of signal peptide cleavage for the US11 molecule lacking its transmembrane/cytoplasmic tail region (US11-180) is significantly delayed when compared with that seen for wild-type US11 (Figure 5). Replacement of the US11 signal sequence for that of H-$2K^b$ results in rapid processing of US11 lacking the transmembrane segment, such that signal sequence-containing forms are no longer detected. The unprocessed US11-180 polypeptide is probably in an orientation unfavorable for signal peptide cleavage, and the presence of the US11 transmembrane anchor is clearly required for efficient signal peptide processing (Figure 8).

How can the US11 transmembrane anchor accelerate removal of the US11 signal peptide? The transmembrane domain may interact with the signal peptide and position the signal peptide to facilitate access to the cleavage site. Alternatively, the transmembrane anchor may interact with the SPC and enhance recognition of the US11 signal peptide for reasons of physical proximity. While the specificity of signal peptide cleavage is appreciated in terms of the minimum sequence requirements, cleavage itself is a highly regulated process, the dynamics of which are not well understood. The non-catalytic subunits of the SPC have been cloned and isolated, yet their function remains to be determined. Our results show that regulation of signal peptide cleavage may involve *cis*-acting elements within the polypeptide that act at considerable distance

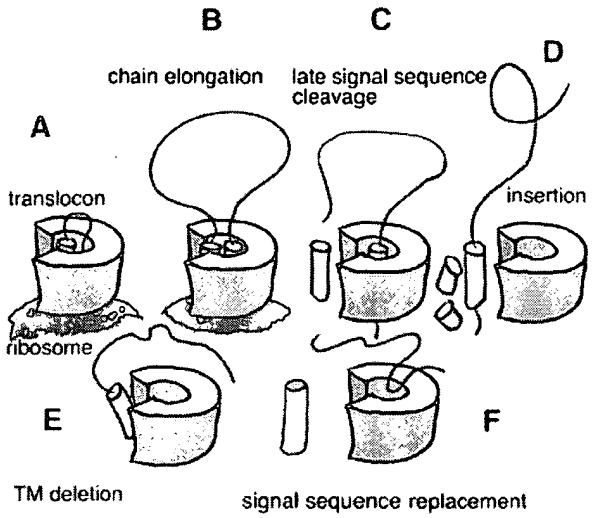


Fig. 8. Model of HCMV US11 signal peptide cleavage. (**A**) The signal peptide (pink) is inserted into the translocon, followed by (**B**) chain elongation of the US11 nascent polypeptide. (**C**) Upon completion of US11 translation, the US11 transmembrane segment (blue) may interact with the signal peptide to delay signal peptide cleavage. (**D**) Upon cleavage of the signal peptide, the US11 molecule inserts into the lipid bilayer; the signal peptide itself may be cleaved further by signal peptide peptidase. (**E**) The signal peptide of a truncated US11 molecule that lacks its transmembrane region and cytoplasmic tail (US11-180) is cleaved inefficiently from the nascent polypeptide. (**F**) Replacement of the US11 signal peptide in US11-180 with the H-2K^b signal peptide (green) results in efficient processing. The US11 transmembrane domain may position the signal peptide in an orientation favorable for cleavage.

from the actual cleavage site. Such elements could perhaps interact with the non-catalytic subunits of signal peptidase.

Immunoelectron microscopy, the maturation status of its single N-linked glycan and the kinetics with which it catalyzes accelerated destruction of class I molecules all place US11 in the ER. The ER environment of the US11 signal peptide may help determine the unusual signal peptide cleavage pattern that we observe. The site of signal peptide cleavage is in the ER and is postulated to be in close proximity to the translocon (Kalies *et al.*, 1998). An intrinsic feature of the US11 signal peptide, more specifically the c-region of the signal peptide, may dictate an association with complexes within the ER as judged from the observed cleavage in detergent extractability (Figure 4 and D.Tortorella and H.L.Ploegh, unpublished data). Shortly after signal peptide cleavage, the recovery of the processed form of US11 increases over the chase period. We suggest that these early biosynthetic forms of US11 may reside in specialized regions of the ER.

To address an issue more peripheral to the central claims of this study: is the cleavage pattern of US11's signal sequence related to US11-induced class I degradation? The signal peptide of the chimeric molecule US11–K$^b_{18-215}$ is cleaved rapidly and this molecule readily supports class I destruction (D.Tortorella and H.L.Ploegh, unpublished data). Therefore, the identity of the US11 signal peptide itself is not essential for the ability of US11 to accelerate class I degradation. The signal peptide of the chimera US11/A2$_{TM/CT}$ is also cleaved rapidly, but class I heavy chains are not degraded in US11/A2$_{TM/CT}$-expressing cells (D.Tortorella and H.L.Ploegh, unpublished data). Deletion of US11's cytoplasmic tail does not abolish degradation of class I heavy chains (D.Tortorella and H.L.Ploegh, unpublished data), and consequently the identity of the transmembrane segment of US11 should be considered essential to its function.

If our interpretation is correct, then perhaps the interaction of the US11 signal peptide and US11 transmembrane segment would help keep the Sec61 complex and its accessories in a configuration that allows recruitment of the class I heavy chains to the translocon. The recorded efficiency of US11-mediated dislocation suggests that the process is tightly linked, temporally and perhaps physically, to protein translocation into the ER. Thus, close proximity of US11 to the translocation apparatus and efficient gating of the protein channel might account for the speed of the dislocation reaction. Ultimately, this aspect must be related to the properties of US11 itself. The unusual maturation of US11, as described here, may turn out be an important aspect of how the dislocation apparatus is put in place.

Materials and methods

Cell lines and antibody

U373-MG astrocytoma cells transfected with the US11-215 cDNA were prepared as described (Jones *et al.*, 1995; Kim *et al.*, 1995) and cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) and 5% calf serum. US11-201, US11-180, US11K$^b_{18-215}$ and US11/A2 cells were maintained in DMEM supplemented with 5% FCS, 5% calf serum and 0.5 mg/ml geneticin (Gibco, Fredrick, MD). The human embryonic kidney cell line (HEK-293) was maintained in DMEM supplemented with 5% FCS and 5% calf serum. The anti-US11 serum was generated by immunizing rabbits with fragments of US11 (amino acids 18–36, 104–122 and 194–210) conjugated to keyhole limpet hemocyanin (Story *et al.*, 1999). The anti-class I heavy chain serum was generated by immunizing rabbits with the bacterially expressed lumenal fragment of HLA-A2 and HLA-B27 heavy chains (Tortorella *et al.*, 1998). The anti-β_2m serum was generated by immunizing rabbits with bacterially expressed human β_2m.

Metabolic labeling of cells and pulse–chase analysis

Cells were detached by trypsin treatment, followed by starvation in methionine/cysteine-free DMEM for 45 min at 37°C. Cells were metabolically labeled with 500 μCi of [^{35}S]methionine/cysteine (1200 Ci/mmol; NEN-Dupont, Boston, MA)/ml at 37°C for the times indicated. In pulse–chase experiments, cells were radiolabeled as above and were chased for the times indicated in DMEM containing non-radiolabeled methionine (2.5 mM) and cysteine (0.5 mM). Cells were then lysed in NP-40 lysis buffer (10 mM Tris pH 7.8, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% NP-40) supplemented with 1.5 μg/ml aprotinin, 1 μM leupeptin, 2 mM phenylmethylsulfonyl fluoride (PMSF) followed by immunoprecipitation (see below). For cells lysed in 1% SDS, the SDS concentration was adjusted, prior to immunoprecipitation, to 0.063% with the NP-40 lysis mix.

Immunoprecipitation

Following cell lysis, cell debris was removed by centrifugation at 10 000 *g* for 10 min. Non-specific binding proteins were removed from the cell lysates by the addition of 3 μl/ml normal rabbit serum, 3 μl/ml normal mouse serum and formalin-fixed, heat-killed *Staphylococcus aureus* for 1 h at 4°C. Immunoprecipitation was performed by incubation with antiserum for 45 min at 4°C, followed by the addition of *S.aureus* for 45 min at 4°C. The pelleted *S.aureus* were washed four times with washing buffer (0.5% NP-40 in 50 mM Tris pH 7.4, 150 mM NaCl and 5 mM EDTA). The pellet was resuspended in SDS sample buffer (4% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue in 62.5 mM Tris pH 6.8) and the released materials were subjected to 12.5% SDS–PAGE.

cDNA, transfection and Endo H digestion

The cDNA of full-length US11 was cloned from the AD169 HCMV genome using the following primers: 5′ primer, CCGCTCCGAGCGGCGTCGACACCACCATGGAACCTTGTAATGCTTATTCTAGC; 3′ primer, GCTCTAGAGCTCACCACTGGTCCGAAAACATCCAG. The US11 cDNA was cloned into the eukaryotic expression vector pcDNA 3.1 (Invitrogen, Carlsbad, CA) using the *Xho–Xba* restriction site in its polylinker region. US11-180 was subcloned from US11 (pcDNA3.1). The chimeric molecules: US11/A2$_{TM/CT}$ [US11(amino acids 1–178)/HLA-A2(amino acids 307–365)]; US11–K$^b_{5-215}$ [H-2K^b(amino acids 1–5)/US11(amino acids 5–215)]; US11–K$^b_{13-215}$ [H-2K^b(amino acids 1–16)/US11(amino acids 13–215)]; US11–K$^b_{18-215}$ [H-2K^b(amino acids 1–21)/US11(amino acids 18–215)]; and US11–K$^b_{18-180}$ [H-2K^b(amino acids 1–21)/US11(amino acids 18–180)] were generated by initially cloning the desired fragment followed by ligation of two of the respective fragments. Using primers specific to the ends of the ligated molecule, it was recloned and inserted into pcDNA3.1. A liposome-mediated transfection (Lipofectamine, Gibco, Fredrick, MD) protocol was performed as described by the manufacturer (4 μg of DNA/20 μl of lipofectamine/10 cm dish of cells). Endo H (New England Biolabs) digestion was performed as described by the manufacturer.

Gel electrophoresis

SDS–PAGE and fluorography were performed as described (Ploegh, 1995). For N-terminal sequencing, the immunoprecipitated US11 protein was resolved by SDS–PAGE and transferred to a PVDF membrane (0.22 μm pore size) in transfer buffer (48 mM Tris-base, 39 mM glycine, 0.037% SDS, 20% methanol) using a semi-dry blotting apparatus (Buchler Instruments, Kansas, MO).

N-terminal sequence analysis

The PVDF membrane that contained the polypeptide of interest was subjected to automated Edman degradation using an Applied Biosystem Protein Sequencer, Model 477, using ATZ chemistry, at the Biopolymers Laboratory at MIT, Center for Cancer Research. The fractions from each degradation sequencing cycle were collected and counted by liquid scintillation spectrometry.

Na_2CO_3 treatment

US11-215 and US11-180 cells were metabolically labeled for 15 min and then washed twice in 50 mM Tris pH 7.5, 250 mM sucrose (homogenization buffer). The cells were resuspended in homogenization buffer and broken by vortexing in the presence of 106 μm glass beads. The homogenate was centrifuged at 1000 *g* for 5 min; the pellet fraction was resuspended in NP-40 lysis mix (see above) and the supernatant was treated with Na_2CO_3 (100 mM final) for 30 min at 4°C (Fujiki *et al.*, 1982). The Na_2CO_3-treated samples were centrifuged at 150 000 *g* using a TLA 100.2 rotor in a Beckman centrifuge. The 150 000 *g* high pH supernatant was adjusted to pH 7 with 1 M HCl and diluted to a final 1× NP-40 lysis mix. The 150 000 *g* pellet was washed twice with homogenization buffer and then resuspended in 1× NP-40 lysis mix. US11 and β_2m were immunoprecipitated from the 1000 *g* pellet, 150 000 *g* supernatant and the 150 000 *g* pellet with the respective antibody.

Acknowledgements

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